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Cold stress effects on organelle ultrastructure in polar Caryophyllaceae species

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Abstract: This study investigated leaf mesophyll cells of Caryophyllaceae plants growing in polar regions – *Cerastium alpinum* and *Silene involucrata* from the Hornsund region of Spitsbergen island (Svalbard Archipelago, Arctic), and *Colobanthus quitensis* from the Admiralty Bay region on King George Island (South Shetland Islands, West Antarctic). Ultrastructural changes were analyzed in mesophyll protoplasts of plants growing in natural Arctic and Antarctic habitats and plants grown in a greenhouse, including plants exposed to short-term cold stress under semi-controlled conditions. Cell organelles of plants growing in natural polar habitats and greenhouse-grown plants were characterized by significant morphological plasticity. Chloroplasts of plants studied in this work formed variously shaped protrusions and invaginations that visibly increased the contact area between adjacent cell compartments and reduced the distance between organelles. *S. involucrata* plants grown under greenhouse conditions, tested by us in this work, were characterized by highly dynamic cell nuclei with single or multiple invaginations of the nuclear membrane and the presence of channels and cisternae filled with cytoplasm and organelles. Crystalline inclusion proteins were observed in the cell nuclei of *C. quitensis* between nuclear membranes and in the direct proximity of heterochromatin. Our study revealed significant conformational dynamics of organelles, manifested by variations in the optical density of matrices, membranes and envelopes, in particular in *C. quitensis*, which could suggest that the analyzed Caryophyllaceae taxa are well adapted to severe climate and changing conditions in polar regions.

Key words: Arctic, West Antarctic, *Cerastium alpinum*, *Colobanthus quitensis*, *Silene involucrata*, cold stress, organelles.

Introduction

Vascular plants inhabiting polar regions have developed specific adaptive traits that enable them to respond to changes in environmental conditions, includ-

ing changes that take place within a short period of time (Day *et al.* 1999; Lütz 2010; Parnikoza *et al.* 2011). Analyses of flowering plants growing in their natural habitats revealed the stability of the photosynthetic apparatus during the day/night cycle and throughout the growing season. Severe conditions in the Antarctic probably induced genetic changes that maximized the photosynthetic efficiency of the studied plants (Bravo *et al.* 2007); therefore, low temperature is not a limiting factor. The optimal temperature for photosynthesis is 13°C in *Deschampsia antarctica* E. Desv. and 19°C in *Colobanthus quitensis* (Kunth) Bartl., but the efficiency of photosynthesis in both species can exceed 30% at a temperature of 0°C (Xiong *et al.* 1999; Casanova-Katny *et al.* 2006). The above indicates that the analyzed taxa are able to effectively assimilate carbon at low temperatures (Perez-Torres *et al.* 2006) and have developed effective mechanisms to neutralize reactive oxygen species (Perez-Torres *et al.* 2004).

One of the mechanisms by which polar plants protect themselves against low temperature is the synthesis of various stabilizing proteins whose presence in plant cells has been demonstrated by various studies (Reyes *et al.* 2003; Bravo and Griffith 2005; Olave-Concha *et al.* 2005; John *et al.* 2009). The majority of those studies investigated *C. quitensis* and *D. antarctica*, native species of West Antarctic (Parnikoza *et al.* 2011). The *D. antarctica* genome probably contains more than ten genes encoding dehydrins, proteins that maintain the cell's physiological integrity under stress. Dehydrins were observed mostly in tissues where the process of ice crystal formation is initiated – the epidermis and bundle sheaths of *D. antarctica* (Olave-Concha *et al.* 2005).

Anti-freeze proteins (AFPs) constitute a specific group of proteins that prevent the formation of ice crystals in the tissues of *D. antarctica* (Bravo and Griffith 2005). AFPs inhibit the growth and modify the shape of ice crystals in the apoplast, which slows down protoplast dehydration and increases the plant's resistance to frost (Doucet *et al.* 2000; Griffith and Yaish 2004). Bravo and Griffith (2005) observed that AFP accumulation is a part of the mechanism of low-temperature tolerance in *D. antarctica.* Ice recrystallization inhibition proteins (IRIPs) play a similar role in the apoplast of the analyzed species (John *et al.* 2009). No such mechanisms were observed in *C. quitensis* plants (Bravo and Griffith 2005) that avoid freeze damage through tissue cooling (Bravo *et al.* 2001).

Heat shock proteins (HSPs) with molecular weight of 70 kDa were also identified in *D. antarctica* (Reyes *et al.* 2003). The main role of HSPs is to prevent protein aggregation.

Studies of cold-sensitive plants such as beans and cold-resistant plants, such as peas revealed, that differences in the protein and lipid composition of thylakoid membranes determine plants' sensitivity to low temperatures (Garstka *et al.* 2007). The subcooling of pea and bean plants at low night-time temperatures and optimal day-time temperatures induced considerable changes in the chloroplast structures of both cold-sensitive and cold-resistant plants, but the observed processes were

much slower in pea chloroplasts. Cold stress led to changes in the structure of protein-chlorophyll photosynthetic complexes, in particular in the proportions of antenna protein isoforms in both peas and beans (Rumak *et al.* 2010). At low temperatures, the concentrations of hydrogen peroxide and nitric oxide were higher in the cells of bean than pea plants. The observed changes clearly indicate that the structure of chloroplasts is closely linked to their function (Kolodziejek *et al.* 2007).

Cold stress is often accompanied by dehydration of plant cells due to water freezing in tissues. Significant water loss is manifested by the degradation of internal chloroplast and mitochondrial membranes and the gradual breakdown of the mitochondrial stroma and matrix (Lopez-Carbonell *et al.* 1994).

Physiological responses to environmental stressors are manifested in the ultrastructure of cell organelles. Chloroplasts are the most damage-sensitive organelles (Kratsch and Wise 2000). The shape and content of chloroplasts and the structure of their internal membranes are influenced by environmental stressors.

Studies involving microscopic analyses revealed significant plasticity of cell organelles in several species of polar and alpine flowering plants (Lütz *et al.* 2012), which was manifested by the examined taxa's ability to produce various surface deformations. The evaluated plants contained chloroplasts with pockets filled with cytoplasm, vesicles and organelles, chloroplasts with differently sized protrusions containing stroma and thylakoid systems (Giełwanowska 2003; Giełwanowska *et al.* 2005; Giełwanowska and Szczuka 2005), chloroplasts with vast areas that were free of thylakoids and were filled only with stroma (Holzinger *et al.* 2007a, b) as well as thin and long protrusions – stromules (Shaw and Gray 2011). Similar chloroplasts with numerous invaginations filled with mitochondria were observed in the cells of Antarctic lichen algae (Giełwanowska and Olech 2012).

Dense stroma, a well organized system of internal membranes and differences in their conformational state are the characteristic features of mitochondria in the mesophyll of flowering plants native to the Antarctic (Giełwanowska *et al.* 2005).

Very large peroxisomes with a dense matrix were noted in the direct proximity of mitochondria and chloroplasts in mesophyll cells of polar plants and high-alpine vascular plants (Lütz and Engel 2007). In Poaceae plants of polar regions, peroxisomes are accompanied by crystalline protein structures in the matrix (Giełwanowska *et al.* 2005). Peroxisomes are usually spherical, but variously shaped and elongated peroxisomes were also reported (Muench and Mullen 2003). The existing research failed to demonstrate whether the noted protrusions are indirect structures created during biogenesis or structures that participate in metabolite exchange and increase the contact area between cell organelles, as is the case with chloroplast deformations – stromules (Muench and Mullen 2003).

The nuclei of mesophyll cells in flowering plants are generally spherical, but they can take on various shapes. Different visualization techniques have been used to analyze atypical characteristics of cell nuclei, including their ability to create grooves and invaginations (Singh *et al.* 1998; Collings *et al.* 2000; Meier 2001).

This study examined the ultrastructure of organelles in the mesophyll cells of Caryophyllaceae plants: *Cerastium alpinum* L. and *Silene involucrata* (Cham. *et* Schltdl.) growing in the Arctic and *Colobanthus quitensis* native to West Antarctic. Leaf mesophyll cells from plants growing in natural habitats were chemically preserved at harvest. This study also analyzed leaf mesophyll cells from the same plant species grown in an experimental greenhouse, including plants exposed to short-term cold stress.

Materials and methods

Plant material. — *Colobanthus quitensis*, *Cerastium alpinum* and *Silene involucrata* plants were harvested during polar expeditions organized in 2008–2010 in the region of the *Stanisław Siedlecki* Polish Polar Station in Hornsund, Spitsbergen (77°00' N and 15°33' E) and the *Henryk Arctowski* Polish Antarctic Station on King George Island (62°09' S and 58°28' W, South Shetland Islands). The plants were chemically preserved and embedded in epoxide resin at harvest.

Plant cultivation. — Seeds and live plants were secured and transported to Olsztyn. Plant specimens were planted in pots with garden soil, and seeds were sown in soil and grown at approximately 20°C in the experimental greenhouse of the Wydział Biologii i Biotechnologii Uniwersytetu Warmińsko-Mazurskiego w Olsztynie (Faculty of Biology and Biotechnology of the University of Warmia and Mazury in Olsztyn). Plant tissue was sampled for analysis. In this study, those plants are referred to as greenhouse plants grown at a temperature of 20°C. Selected specimens were additionally grown in a climate test cabinet (Nüve Climate Test Cabinet, TK 252) at a temperature of 8/4°C (day/night) and 16h/8h (day/night) photoperiod. Tissue samples were also collected from the above plants for analysis. In this study, those plants are referred to as plants grown at a temperature of 8/4°C.

Determining the location of insoluble polysaccharides. — Insoluble polysaccharides were determined by the Periodic acid-Schiff (PAS) reaction (Pearse 1985) in leaf blade segments fixed in Carnoy's solution (96% ethanol and acetic acid v/v, 3:1) at room temperature for 12 hours. The specimens were dehydrated in a graded series of ethanol and were embedded in Poly Bed 812 epoxide resin. Semi-thin sections (1.5 μm thick) were prepared in the microtome (Leica Ultracut R) using diamond knives (Diatome). The specimens were placed on slides and mounted with a drop of glycerin. They were analyzed under the Nikon Eclipse 80i light microscope with compatible hardware and software (NIS ELEMENTS) for digital image recording.

Analyses of leaf anatomy and ultrastructural analyses of mesophyll cells. — Specimens for analysis under a light microscope (LM) and a transmission elec-

tron microscope (TEM) were fixed in 3.5% glutaraldehyde solution in phosphate buffer for 24 hours at room temperature, followed by secondary fixation in 2.5% osmium tetroxide solution. The specimens were rinsed and dehydrated in a graded series of alcohols and acetone and were embedded in Poly Bed 812 epoxide resin (Polyscience). Microtome sections were prepared in a microtome (Leica Ultracut R) using diamond knives (Diatome). Semi-thin sections (1.5 μm thick) were placed on slides and stained with toluidine blue and azure B. They were analyzed according to the procedure applied to specimens subjected to the PAS reaction. Ultra-thin sections (60–90 nm thick) were mounted on nickel grids with 300 mesh squares. Directly before examination, saturated aqueous uranyl acetate solution and lead citrate were added to impart contrast to the specimens. The specimens were examined and electronograms were obtained simultaneously under two transmission electron microscopes – JEOL JEM 100S and JEOL 1400. JEOL JEM 100S supports analog image recording, whereas JEOL 1400 is equipped with hardware and ITEM software for recording data files.

Analysis of cell organelles responses to cold stress. — Plants grown in pots in a greenhouse (one plant per pot, two replications) were transferred to the laboratory and acclimatized. After 24 hours of incubation at room temperature (18–20°C), the pots were placed in the Heraeus BK6160 incubator (Kendro Laboratory Products) with constant temperature of 20°C and 20 h/4 h (day/night) photoperiod. After 24 hours, the plants were subjected to cold stress at 4°C for 48 hours. After 1, 4 and 12 hours, 3–4 mm long leaf segments (central part of the leaf blade of the second or third leaf) were removed and fixed. After 48 hours of exposure to cold stress, the plants were allowed to equilibrate at 20°C, and the last tissue samples were collected after 24 hours at 20°C. Tissue samples collected from unstressed plants at 20°C served as the control.

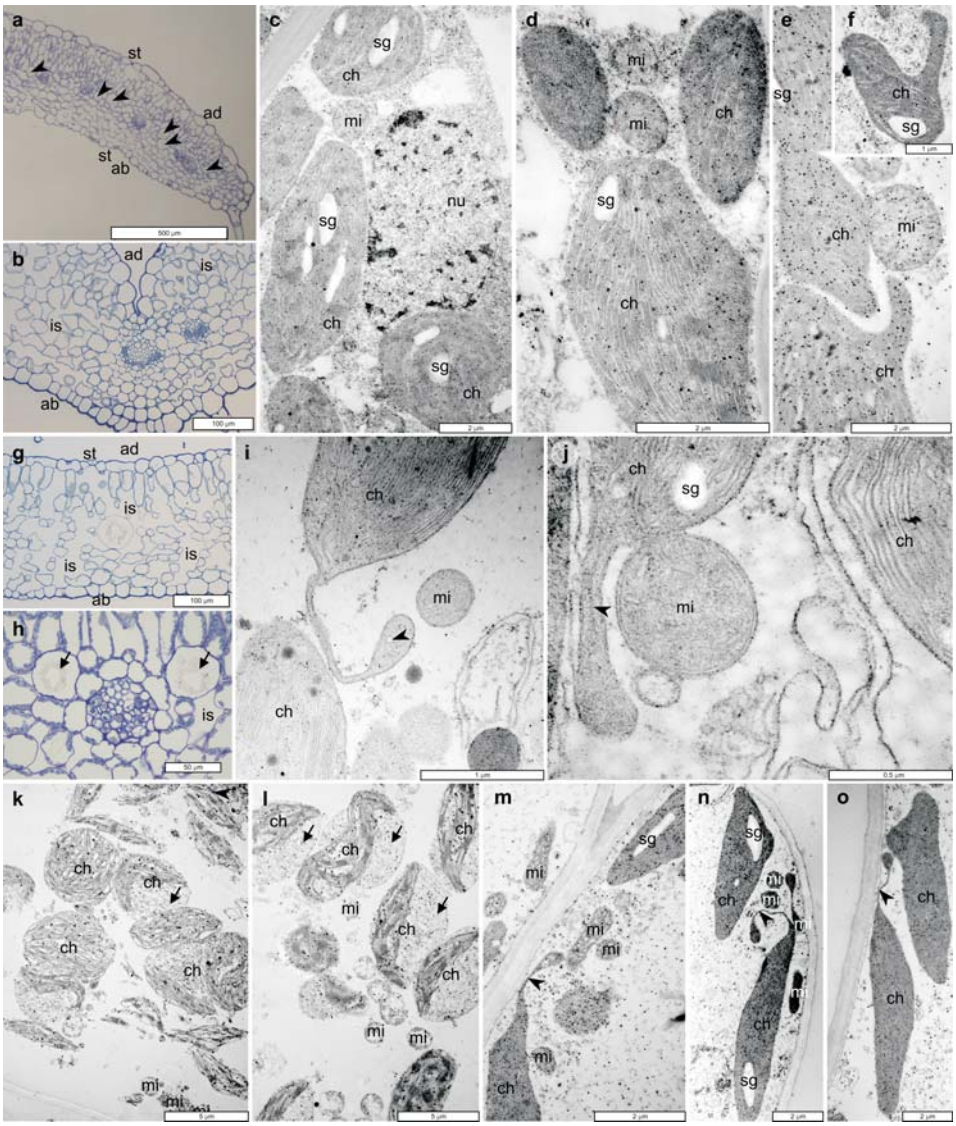
Results

Leaf anatomy of Caryophyllaceae plants. — The mesophyll of bifacial leaves of the analyzed species is composed of palisade and spongy cells (Figs 1a–b, g, 2a and 4a). Mesophyll cells of *C. alpinum* harvested in the Arctic had a regular shape (Fig. 1a, h). In greenhouse-grown plants, palisade parenchymal cells were mostly elongated, whereas spongy parenchymal cells were mostly isodiametric (Fig. 1b, g). Mesophyll cells of *S. involucrata* harvested from greenhouse conditions (Fig. 4a, c) had a similar shape, but parenchymal cells of the bundle sheath and the adjacent mesophyll cells near vascular bundles were irregular in shape (Fig. 1b). Large intercellular spaces were observed between parenchymal cells in peripheral and central segments of the leaf blade in all analyzed Caryophyllaceae plants (Figs 1a–b (is), g (is), h (is), 2a (is) and 4a–c (is)).

Collateral vascular bundles in all analyzed plants were noted in the central part of the leaf along the boundary between palisade and spongy parenchymal layers. Subject to species, stage of leaf development and distance from the leaf base, four to six vascular bundles were reported in *Colobanthus quitensis* (Fig. 2a), and up to 10 vascular bundles were observed in *Cerastium alpinum* (Fig. 1a) and *Silene involucrata* (Fig. 4a). Vascular bundles were surrounded by parenchymatic sheaths with closely adhering cells. Those cells had regular (Fig. 1b,h) or irregular shape (Fig. 4b), and they did not contain starch. Varied amounts of starch granules were noted in the chloroplasts of the remaining mesophyll cells of analyzed polar plants. Starch granules were abundant in the leaf parenchyma of *C. alpinum* harvested in the Arctic (Fig. 1h), which was confirmed by TEM images (Fig. 1c–f), in leaf mesophyll tissue of *C. quitensis* plants harvested in the Antarctic (Fig. 2d, h) and in greenhouse-grown *S. involucrata* plants (Fig. 4b). The remaining greenhouse-grown plants contained far less starch (Figs 1j, m–o). Large amounts of insoluble, PAS-positive polysaccharides were determined in the cell walls of all leaf tissues, in particular in external walls that come into contact with abaxial and adaxial epidermis and in vascular bundles. Idioblasts with PAS-negative druse crystals were observed between vascular bundles (Figs 1a, arrowheads, h, arrows, 2a and 4c, arrows).

The presence and ultrastructure of cell organelles. — In leaf cells of Caryophyllaceae plants growing in natural Arctic and Antarctic habitats, fixed immediately after harvest, the organelles were very densely packed (Fig. 1c–f), whereas much looser organelle arrangement was noted in tissue specimens collected from plants grown in a greenhouse under varied conditions due to the four-, fivefold larger size of their cells. In several cases, cell organelles from the leaf cells of *Cerastium alpinum* grown in the Arctic region were tightly clustered, and their surfaces were deformed. Chloroplasts with a regular surface (Fig. 1c–d) and chloroplasts forming invaginations and protrusions (Fig. 1e–f) were observed in mesophyll cells of *C. alpinum* plants harvested and fixed in the Arctic. In mesophyll cells of plants grown in a greenhouse under varied temperatures, chloroplasts formed characteristic shorter or longer protrusions that had equal width or were wider at the ends and were filled with stroma and thylakoids (Fig. 1i–j, arrow-

Fig. 1. Anatomical structure of leaf blades and cell organelles in the cytoplasm of mesophyll leaf cells of *Cerastium alpinum* grown in the Arctic region (a, h, c–f). Ultrastructure of the cell organelles of *C. alpinum* grown under greenhouse conditions (b, g, i–o). **a**. Section of the leaf blade with varied adaxial (ad) and abaxial (ab) epidermis and numerous stomata (st). Numerous collateral vascular bundles are visible along the boundary of palisade and spongy mesophyll layers. Idioblasts with crystals (druse, arrowheads) between vascular bundles. Semi-thin sections were stained with toluidine blue and azure B. **b**, **g**. A section of central (b) and lateral (g) region of the leaf blade. Regular, elongate palisade cells and mostly isodiametric spongy mesophyll cells can be seen. Mesophyll cells are separated by extensive intercellular spaces (is). **h**. Lateral vascular bundles surrounded by a parenchymatic bundle sheath. High concentrations of starch grains are visible in three chloroplasts in both palisade and spongy mesophyll layers. Starch grains are not observed only in bundle sheath chloroplasts. Cells containing crystal chambers are found in the proximity of bundle sheats (arrows). →



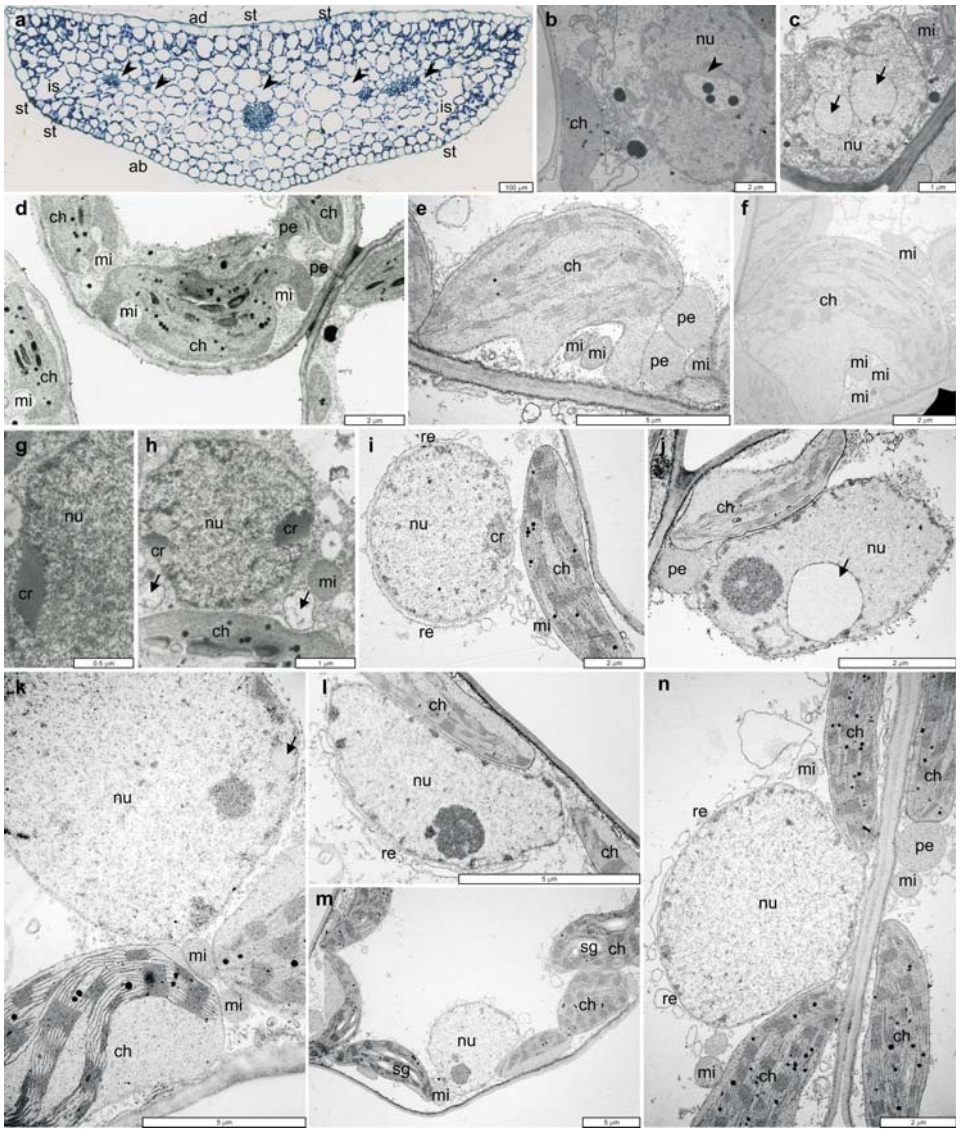
c–**f**. The cell nucleus (nu), mitochondria (mi) and chloroplasts (ch) are packed tightly together. Deformed chloroplasts are shown in e and f. In c the cell nucleus is packed tightly together with the chloroplasts. Nearly all chloroplasts contain starch grains. **i**, **j**. Atypically shaped chloroplasts and mitochondria in leaf mesophyll cells of plants grown at a variable temperature of 8/4°C. Chloroplasts with starch (j, sg) form characteristic protrusions with thylakoid (arrowheads). Plastoglobules are present in chloroplasts. **k** and **l**. Destructive changes in chloroplasts (ch) and mitochondria (mi) under greenhouse conditions. Short and thick chloroplasts with visibly dilated thylakoid lumina and large sections containing stromal parts without thylakoids (arrows). Mitochondria with disorganized internal membrane structures. **m**–**o**. Destructive changes in chloroplasts (ch) and mitochondria (mi) of plant incubated at the temperature of 4°C for 12 hours. Chloroplasts contain infrequent large starch grains, dense osmiophilic stromal parts, and they form protrusions with thylakoids (m, arrowheads). The lumina of internal membrane vesicles in the mitochondria (mi) are dilated.

heads). Plants subjected to short-term cold stress (4°C for 12 hours) contained chloroplasts with long protrusions that were wider at the ends and featured visible thylakoid lumina (Fig. 1m–o, arrowheads). Stromules of varied length were formed on the side of the cell wall and often closely adhered to it. The leaf cells of greenhouse-grown plants contained normal mesophyll cells as well as cells with degenerating chloroplasts and mitochondria (Fig. 1k–l).

Chloroplasts with deformed surfaces were noted in mesophyll cells of *Colobanthus quitensis* plants harvested from their natural habitat in the Antarctic and grown in a greenhouse. Deformations had the shape of pocket invaginations that closely surrounded single mitochondria (Fig. 2d), larger invaginations that enclosed several mitochondria (Fig. 2e–f) or longer protrusions containing stroma and thylakoids (Fig. 3k). Extensive thylakoid-free areas were visible in the chloroplasts of plants grown at both constant and varied temperature (Figs 2d, i–j and 4d–e). The chloroplasts of *C. quitensis* plants contained starch globules and lipid plastoglobules, and lipid drops were particularly abundant in the material collected in the Antarctic (Fig. 2d, h).

The mesophyll cells of the analyzed plants contained numerous mitochondria in the proximity of chloroplasts. The mitochondria had a spherical shape in the mesophyll cells of *Cerastium alpinum* plants incubated at the temperature of 4°C for 12 hours, and they were finger-shaped or elongated in the mesophyll cells of this species plants subjected to cold stress (Fig. 1m (mi), n (mi)). In the remaining cells, the mitochondria were of various shapes – from spherical, which were most frequently observed in the mesophyll cells of *C. quitensis* plants harvested from the Antarctic (Fig. 2c–d (mi), h (mi)), to elongated and finger-shaped, which were noted mostly in plants grown under constant (20°C) and varied (8/4°C) temperature (Fig. 2g–k (mi)). In addition to normally arranged mitochondria, the cytoplasm of *C. quitensis* cells also contained mitochondria with evident signs of degeneration, mostly lysis. Degenerating mitochondria were frequently reported in plants collected in the Antarctic (Fig. 2d (mi)) and in the cells of plants exposed to low temperatures (Fig. 3l–m (mi)).

Fig. 2. Anatomical leaf structure and differentiation of cell organelles in leaf mesophyll cells of *Colobanthus quitensis* grown in the natural habitat of the Antarctic and under greenhouse conditions. **a**. Anatomical structure of a leaf of plant growing in a greenhouse. Stomata (st) are visible in the adaxial (ad) and abaxial (ab) epidermis. Mesophyll cells on the adaxial side of the blade are slightly elongated and contain more chloroplasts, and those on the abaxial side are isodiametric and contain fewer chloroplasts. The system of intercellular spaces (is) in the mesophyll is well-developed. Five vascular bands with various amounts of conducting tissue are visible. Parenchymal bundle sheaths (arrowheads) with a few chloroplasts are also visible. Semi-thin section stained with toluidine blue. Scale = 100 μm. **b**, **c**. Ultrastructure of parts of mesophyll cells of plants grown in the natural habitat of the Antarctic with nuclei located close to the cell wall (c, nu). The irregular surface of the cell nucleus is shown. Protein crystals, individual or paired, in the cell nucleus inside the circular area with lighter content and lipid droplets (b, nu, arrowhead). Circular areas with lighter or darker content inside the cell nucleus surrounded by a membrane (c, arrows) are observed. **d**. Fragments of leaf →



mesophyll cells of plant from Antarctic tundra. Chloroplasts (ch) with starch grains and plastoglobules have numerous pocked-shaped indentations containing mitochondria (mi) with disorganized internal membrane structures. **e**, **f**. Fragments of mesophyll leaf cells of plant grown at a variable temperature of 8/4°C. Chloroplasts (ch) with deformations in the form of swellings. Mitochondria (mi) with well-organized inner membrane structures inside the invaginations and between the large peroxisomes (pe). **g**, **h**. Fragments of cytoplasm with nuclei in leaf mesophyll cells of plants from Antarctic tundra. **i**–**n**. Ultrastructure of mesophyll cells protoplasts of greenhouse plants grown at a temperature of 20°C. Parts of cytoplasm with nuclei located close to the cell wall and heterochromatin in the area of the nuclear membrane. Longitudinally (i, re) and vertically (l, m, n, re) membranes of the endoplasmic reticulum can be observed in the direct vicinity of cell nuclei. Chloroplasts (ch) contain numerous osmiophilic plastoglobules and starch grains (m). Very large peroxisomes (pe, j, n) are tightly packed together with chloroplasts and mitochondria.

The mitochondria of greenhouse-grown *Silene involucrata* plants were characterized by varied conformational dynamics, different matrix density and varied arrangement of the internal membrane system (Fig. 4d–h (mi)).

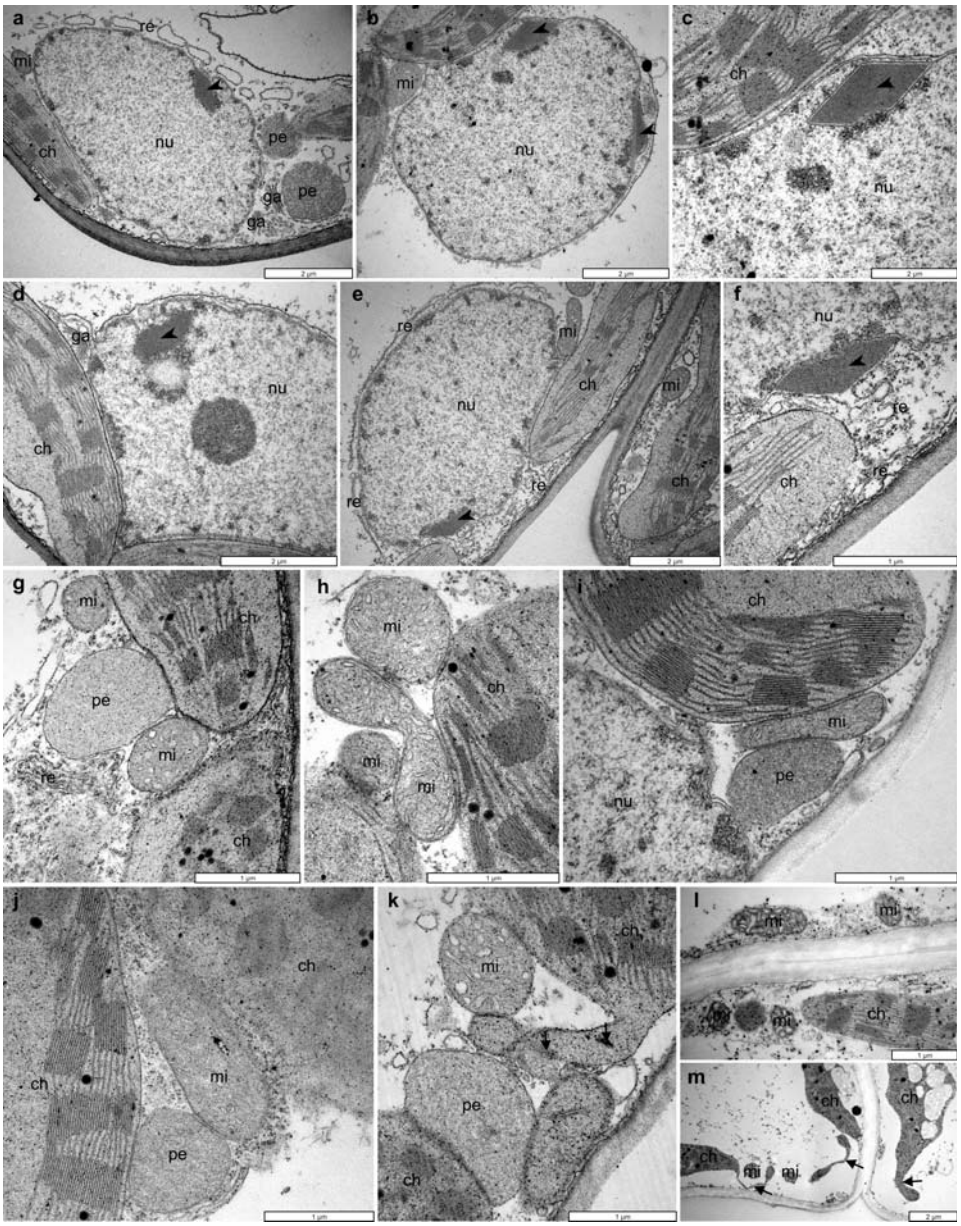
Regular, lens-shaped chloroplasts with a well-organized system of grana thylakoids and stroma thylakoids were observed near the cell wall in mesophyll of *S. involucrata* specimens grown at 20°C and at varied temperature. Small osmophilic plastoglobules and infrequent starch grains were noted in the stroma between grana stacks (Fig. 4f–l). Osmophilic drops (Fig. 4j–k, arrows), whose density was similar to that of cytoplasmic material (Fig. 4l, arrow), were adjacent to the outer membrane of the chloroplast envelope.

The peroxisomes of mesophyll cells of the analyzed Caryophyllaceae plants were significantly larger than chloroplasts and mitochondria. They were densely packed near the remaining organelles, mostly chloroplasts and mitochondria (Figs 2e, j (pe); 3a, g, i–k (pe) and 4k (pe)).

In strongly vacuolized cells of *C. quitensis* plants, spherical and oval cell nuclei were distributed mostly in the cytoplasm along the cell wall, similarly to other organelles. The cell nuclei of the discussed species are characterized by low levels of heterochromatin that is distributed mostly in peripheral regions near the internal nuclear envelope (Fig. 2b–c, g–n (nu) and 3a–e (nu)). The membranes of channels and endoplasmic reticulum cisternae stretched along the outer membrane of the nuclear envelope on the side of the cytoplasm (Figs. 2i (re) and 3a (re), e (re)). Individual (Fig. 2b, arrow; j, arrow; k, arrow) and multiple (Fig. 2c, arrows) spherical areas with brighter or darker material, surrounded by a single-layer membrane, were noted in the region of cell nuclei. Crystalline proteins were visible in the intermembrane space of nuclear envelopes in mesophyll cells of plants harvested from the Antarctic (Fig. 2b (nu), arrowhead, g (cr) and h (cr)) and greenhouse-grown plants (Fig. 3a–f, arrowheads). Both individual and multiple crystalline structures were observed. In some cases, optically bright areas were noted near crystalline proteins in the direct proximity of heterochromatin (Fig. 3d (nu) arrowhead). In *S. involucrata*, the cell nuclei distributed along the cell wall featured numerous protrusions and invaginations filled with cytoplasm and vesicles, organelles and cell material (Fig. 4d–e (nu) and h–i (nu)).

Degenerative changes in organelles were observed in selected cells of *Cerastium alpinum* and *Colobanthus quitensis* plants grown in a greenhouse and subjected to short-term cold stress (4°C). Mitochondria with dilated lumina of the internal membrane system or completely disorganized systems of internal and external membranes were reported (Figs 2d (mi), 3f, (mi) and 4l (mi)). Those cells also contained chloroplasts with atypical features. Chloroplasts contained large, thylakoid-free ar-

Fig. 3. Ultrastructure of mesophyll cell protoplasts of *Colobanthus quitensis* grown under laboratory conditions, at 20°C and at variable temperature, 8/4°C (i) and at 4°C for 12 hours (l, m). **a–f**. Protein crystals (arrowheads) in the nuclei (nu). Characteristic protein crystals, individual and paired, can be →



observed in the nuclear intermembrane space. The nucleus remains in close contact with organelles, mostly chloroplasts (ch), mitochondria (mi), peroxisomes (pe), endoplasmic reticulum (re) and Golgi apparatus (ga). **g–j**. Chloroplasts (ch), mitochondria (mi) and peroxisomes (pe) are tightly packed together. Peroxisomes are large in comparison with the mitochondria. **k**. Organelles in the cytoplasm of leaf mesophyll cells of *Colobanthus quitensis* grown under variable temperature, 8/4°C. Chloroplast with longer protrusion with parts of lamellar system (arrows). **l**, **m**. Mitochondria with dilated lumina of inner membrane vesicles (l, mi) and disorganized chloroplasts with long protrusions (m, arrows) in plant cell incubated for 12 hours at the temperature of 4°C.

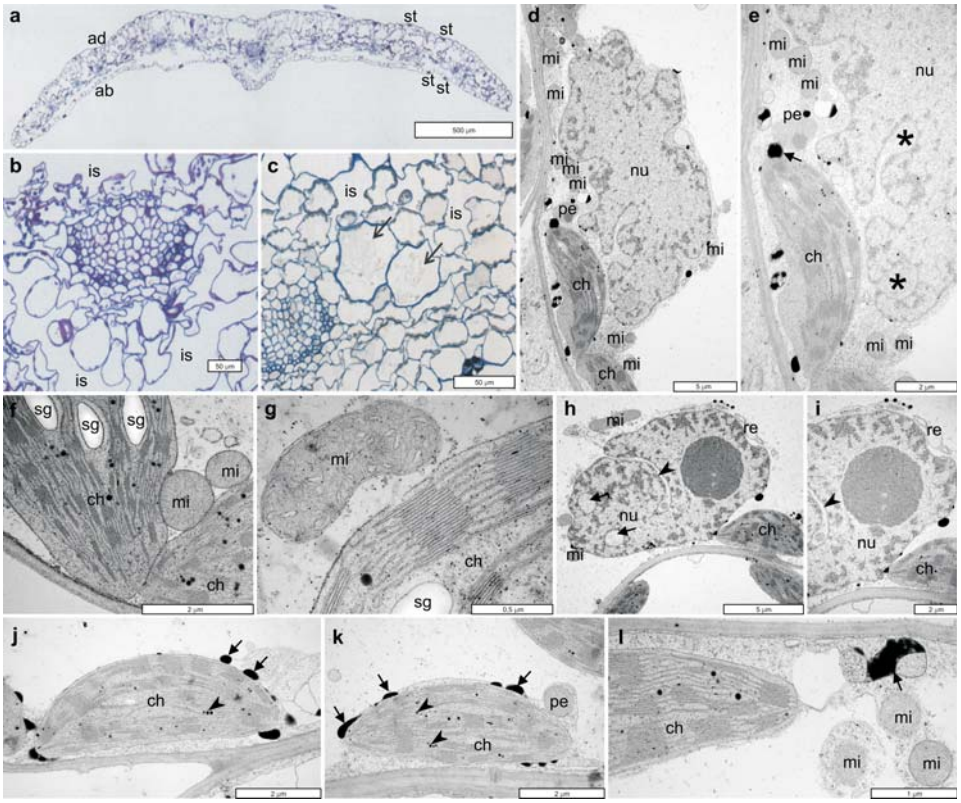


Fig. 4. Anatomical structure of the leaf blade (a–c), insoluble polysaccharides (a–b) and ultrastructure of the organelles in *Silene involucrata* grown under greenhouse conditions. **a**. Cross-sections of the leaf blade. Adaxial (ad) and abaxial (ab) epidermis with numerous stomata. Vascular bundles are visible along the boundary of palisade and spongy mesophyll layers. **b**. Insoluble carbohydrates in cell walls of the mesophyll and vascular bundles and in chloroplasts. Large intercellular spaces (is) can be observed in the mesophyll. **c**. Idioblasts with toluidine blue and PAS negative reactions (arrows) in the mesophyll near the vascular bundles. Large intercellular spaces (is) can be observed in the mesophyll. **d**, **e**. Cell nucleus with numerous protrusions and invaginations filled with cytoplasm with vesicles, organelles and material (stars). Mitochondria with dense matrix (mi), large peroxisomes (pe) and chloroplasts with drops of electron-dense material (arrow) are visible in the cytoplasm near the cell nucleus. **f**, **g**. Cell fragments of *Silene involucrata* grown at a variable temperature of 8/4°C. Chloroplasts with well-organized grana thylakoids contain many large and bright-colored starch grains (sg) and osmiophilic plastoglobules. The organelles are tightly packed together and with variously shaped mitochondria in different conformation states. **h**, **i**. A very large nucleolus and three cytoplasm areas are visible in the cell nucleus – two in cross-section (arrows) and one in longitudinal section (arrowhead). **j**–**l**. Cell fragments of *Silene involucrata* grown at a variable temperature of 8/4°C. Chloroplasts (ch) in the cytoplasm adjacent to the cell wall have a regular surface. They contain regular grana and intergrana thylakoids, infrequent starch grains (sg) and osmiophilic plastoglobules (arrowheads). Osmiophilic droplets are adjacent to the outer membrane of chloroplasts (j, k, arrows) or are found in the cytoplasm (l, arrow).

eas that were filled only with stroma, and laterally distributed thylakoids had dilated lumina (Fig. 1k–l (ch)). Strongly elongated chloroplasts with single, large starch

granules were observed in the cells of plants exposed to a temperature of 4°C for 12 hours. Narrow stromules were visible at the ends of their long axes (Fig. 1m–o (ch) arrowheads). Those changes were characteristic of the degradation of cell organelles and protoplasts.

Discussion

Anatomical responses of polar plants to environmental stressors. — Polar plants adapt to unsupportive environmental conditions by growing in dense patches that prevent heat loss and contribute to a positive energy balance in habitats characterized by low temperatures (Aleksandrova 1983; Block *et al.* 2009). Their adaptive responses are also visible in the structure of epidermal cells, leaf parenchyma cells and vascular bundles. According to many authors, polar plants are xerophytes (Jellings *et al.* 1983; Vieira and Montovani 1995; Romero *et al.* 1999; Mantovani and Vieira 2000; Lewis-Smith 2003; Wielogarski and Karlsen 2007; Lütz 2010; Parnikoza *et al.* 2011). Xerophytic adaptations take place in response to periodic water loss resulting from low temperatures and drought (Alberdi *et al.* 2002).

Xerophytic leaves of *Colobanthus quitensis* are characterized by small size and epidermis comprising thick-walled, densely packed cells and thick cuticle. The plant's specialized floral leaves (Giełwanowska *et al.* 2011) have a unique structure. The epidermis effectively protects cells against mechanical damage, excessive sunlight, harmful ultraviolet radiation, low temperatures and pathogens. In severe polar climates it also protects the shoot against excessive transpiration, wind-borne sand grains and saline aerosol.

Polar plants have also developed various types of epidermal hairs that protect them against chilling winds and excessive radiation (Wielogarski and Karlsen 2007). *Cerastium alpinum* leaves are covered with long mechanical hairs and shorter secretory hairs. Numerous mechanical hairs are also found on the adaxial surface of leaf blades in *Silene involucrata*. *Cerastium uniflorum* Thom. ex Rchb., an alpine species, has developed hairs that densely cover the leaf blade and control light intensity (Lütz and Engel 2007). Epidermal hairs differ in color – from very dark in *Draba* and selected *Ranunculus* species, to lightly colored and completely permeable to sunlight. Willow inflorescences are covered with light-colored hairs that are completely permeable to sunlight, prevent heat loss and maintain internal temperature 15°C higher than ambient temperature (Krog 1955).

The assimilatory parenchyma of the analyzed Caryophyllaceae species is composed of palisade and spongy cells. In *Cerastium alpinum* and *Colobanthus quitensis* plants growing in natural habitats, the majority of leaf cells had regular shape, and large starch grains were visible in chloroplasts. Starch granules in chloroplasts play the role of osmotically neutral storage of assimilatory starch, and

starch hydrolysis preserves cell integrity under exposure to stressors because the produced soluble carbohydrates protect the membranes of thylakoids and other cell structures (Vecchia *et al.* 1998).

Large intercellular spaces were observed between mesophyll cells in the analyzed plants. They formed a continuous system of air channels that facilitate oxygen transport and gas exchange. Intercellular spaces also support the elimination of volatile compounds accumulated in roots, including CO_2, ethylene, organic acids and CH_4, by reverse diffusion (Neue *et al.* 1990). Mesophyll cells with irregular shape were observed near intercellular spaces in semi-thin sections of *C. quitensis* plants. Cell wall invaginations and protrusions increase the area of intercellular spaces and promote gas accumulation (Romero *et al.* 1999). The above changes in the structure and function of mesophyll cells could be induced by long-term stressors. Similar changes in spongy and palisade parenchyma cells and intercellular spaces were described in tomato leaves in response to salinity stress (Sam *et al.* 2003).

Leaf vascular tissue in the examined Caryophyllaceae plants was composed of collateral vascular bundles with very narrow cell lumina. Research has demonstrated that tracheal elements with a small diameter inhibit water freezing (Lütz 2010).

Ultrastructure of mesophyll cells. — Herbaceous plants growing at various latitudes and in different habitats are characterized by significant ultrastructural similarities. The organelles of polar and alpine plants differ only in their plasticity and degree of cooperation, which probably enables those plants to rapidly modify metabolic processes and develop under unsupportive environmental conditions (Lütz *et al.* 2012). Metabolic disruptions induced by environmental factors are manifested in the ultrastructure of cell organelles. Cellular components have varied tolerance to low temperature, dehydration and excessive light exposure. Chloroplasts are the most sensitive organelles, whereas cell nuclei, mitochondria and peroxisomes are characterized by greatest stability (Kratsch and Wise 2000). The ultrastructural organization of organelles determines the response of cells and entire plants to abiotic stress.

Microscopic observations of Caryophyllaceae plants harvested from their natural habitats and grown in a greenhouse under varied conditions revealed tightly packed cytoplasmic organelles. Dense arrangement of organelles is noted in all plants, not only species native to the Arctic and the Antarctic. Chloroplasts, mitochondria and peroxisomes are distributed in close proximity during photorespiration (Körner and Larcher 1988; Larcher *et al.* 1997) and photoinhibition (Holzinger *et al.* 2007a, b).

The most closely adhering chloroplasts and mitochondria were observed in the mesophyll of *C. quitensis* plants growing in a coastal habitat in the Antarctic. Numerous pocket indentations filled with mitochondria were noted in individual chloroplasts (Fig. 2d). In addition to surface deformations, indentations and

invaginations filled with organelles, the chloroplasts of *Cerastium alpinum*, *Colobanthus quitensis* and *Silene involucrata* contained large, thylakoid-free areas. Similar spaces containing only stroma were described in several species of polar and alpine plants, including *Geum montanum* L., *Geum reptans* L., *Oxyria digyna* (L.) Hill. and *Ranunculus glacialis* L. (Lütz 1987; Larcher *et al.* 1997; Holzinger *et al.* 2007a, b; Lütz and Engel 2007; Lütz *et al.* 2012). In the analyzed taxa, chloroplasts formed characteristic shorter or longer protrusions that had equal width or were wider at the ends (Fig. 1i–j arrowheads). In addition to typical stromules filled only with stroma, *C. alpinum* and *C. quitensis* also featured long and narrow protrusions containing membranes and thylakoids (Figs 1i–j, m–o and 3k, m). Stromules that increase the surface area of chloroplasts and come into direct contact with the adjacent cell compartments were previously reported in two species of flowering plants native to the Antarctic, *C. quitensis* and *Deschampsia antarctica* (Giełwanowska *et al.* 2005; Giełwanowska and Szczuka 2005). According to Köhler *et al.* (1997), chloroplast protrusions not only transport molecules, but they can also act as specific receptors that search for areas where the concentrations of ready-to-use molecules are higher. Stromules increase the contact area between chloroplasts and other protoplast elements. The contact area between organelles is expanded or reduced to regulate the rate of macromolecule and micromolecule exchange with the cytoplasm, other organelles and the environment, and this mechanism could play a key role in severe polar climate. The above hypothesis seems to be validated by a recent study by Busch *et al.* (2013) who demonstrated that in wheat and rice cells, the formation of stromules and the distribution of chloroplasts along cell walls increases the contact area between chloroplasts and intercellular spaces. The above facilitates reassimilation of CO_2 that is released into intercellular spaces during photorespiration and normal respiration. Chloroplast stromules are formed on the side of the cell wall and in the direct vicinity of the CO_2 reservoir. Therefore, it can be assumed that the formation of numerous pockets and the establishment of close contacts with mitochondria inside those pockets is driven by the carbon dioxide gradient surrounding chloroplasts. Numerous mitochondria-filled pockets on chloroplast surfaces and close contacts between those organelles have also been observed in the cells of Antarctic lichen algae (Giełwanowska and Olech 2012). Mitochondria cluster around the cell nucleus and trigger hypoxia to minimize damage caused by free radicals (Bereiter-Hahn and Voth 1994; Logan and Leaver 2000).

In previous ultrastructural analyses of *Deschampsia antarctica* cells (Giełwanowska *et al.* 2005), the highest number of pockets, invaginations and protrusions was noted in chloroplasts of plants that grew in coastal areas and were swept by waves during sea storms. In their mesophyll cells, individual chloroplasts simultaneously formed several types of deformations, including long stromules, pockets and invaginations. The above structural arrangement increases the contact area between adjacent organelles and reduces the distance between cell compartments. It aids metabolite flow and eliminates energy expenditure related to long-distance

transport. This energy-saving mechanism could play a vital role in plants growing in extreme climates (Hanson and Sattarzadeh 2011).

Nuclei with highly irregular surfaces were observed in many *Silene involucrata* cells. They formed numerous protrusions and invaginations that were filled with cytoplasm, vesicles and organelles. Spherical areas enclosed by single-layer membrane were observed in the cell nuclei of *C. quitensis* and *S. involucrata*. They resembled nuclear vacuoles of mitotic cells (Sheffield *et al.* 1979; Yi *et al.* 1994) and endoplasmic reticulum elements trapped inside nuclei. It is believed that such structures are a part of the membrane system in the cytoplasm (Singh and Walles 1995; Singh *et al.* 1998). Extended endoplasmic reticulum comprising transverse and longitudinal cisternae was observed in the direct proximity of cell nuclei in *C. quitensis.* It can be assumed that various deformations can participate in the exchange of mRNA and proteins between the nucleoplasm and the cytoplasm, as demonstrated by the location of deformations near the nucleolus and the presence of active cytoplasm with small vesicles in those deformations (Collings *et al.* 2000). The discussed structures probably increase the contact area between the nucleus and the cytoplasm (Meier 2001).

Single and multiple symmetrically distributed crystalline inclusion proteins were observed in the nuclei of mesophyll cells in *C. quitensis* plants growing in the natural habitat and in a greenhouse. The proteins were visible in the intermembrane space of the nuclear envelope, mainly in the proximity of heterochromatin or optically bright areas in the cell nucleus. Electron microscope observations do not support full identification of those structures or their functions. It remains unknown whether those structures had originated in the cell nucleus or whether they were transported to the nucleus from the cytoplasm. Chromatin-stabilizing proteins have been described in numerous studies investigating the production of special cell proteins in response to environmental stressors (Velazquez and Linquist 1984).

In addition to active cells with typical cytoplasmic organization, the mesophyll of Caryophyllaceae plants harvested from natural habitats in the Arctic and the Antarctic, grown in a semi-controlled environment and subjected to short-term cold stress also contained cells with partially or completely damaged chloroplasts and mitochondria (Figs 1k–o and 3l–m). Internal membranes with clearly dilated lumina were observed in mitochondria undergoing various conformational changes. In chloroplasts, the thylakoid system was completely relaxed, and extensive thylakoid-free areas were also noted. Changes in the structure and function of the photosynthetic apparatus inside the internal membrane system are the first symptoms of mild stress; therefore, chloroplasts can be regarded as receptors of environmental stimuli (Kratsch and Wise 2000) and the most sensitive cell organelles. Changes in the conformational state and ultrastructure of organelles in polar Caryophyllaceae plants are most probably adaptive responses to variations in environmental conditions and stimuli. Those mechanisms enable polar plants to grow and survive in unsupportive and changing habitats.

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